Increased Level and Longevity of Protective Immune Responses Induced by DNA Vaccine Expressing the HIV-1 Env Glycoprotein when Combined with *IL-21* and *IL-15* Gene Delivery[1]

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We investigated the ability of a plasmid-derived IL-21 delivered alone or in combination with the *IL-15* gene to regulate immune responses to the HIV-1 envelope (Env) glycoprotein induced by DNA vaccination. Mice were injected with the gp140ΔCFI$_{HXB2/89.6}$ vector expressing a modified Env glycoprotein with C-terminal mutations intended to mimic a fusion intermediate, in which the most divergent region encoding the variable V1, V2, and V3 domains of CXCR4-tropic HxB2 virus was replaced with the dual-tropic 89.6 viral strain. Using a recombinant vaccinia virus expressing 89.6 Env glycoprotein (vBD3) in a mouse challenge model, we observed that IL-21 plasmid produced sustained resistance to viral transmission when injected 5 days after DNA vaccination. Moreover, IL-21 in a synergistic manner with IL-15 expression vector augmented the vaccine-induced recall responses to the vBD3 challenge compared with those elicited by immunization in the presence of either cytokine alone. The synergistic combination of IL-21 and IL-15 plasmids promoted expansion of CD8$^+$CD127$^+$ memory T cell pools specific for a subdominant HLA-A2-restricted Env$_{121–129}$ epitope (KLTPLCVTL). Our results also show that coimmunization with IL-21 and IL-15 plasmid combination resulted in enhanced CD8$^+$ T cell function that was partially independent of CD4$^+$ T cell help in mediating protection against vBD3 challenge. Furthermore, the use of *IL-21* and *IL-15* genes was able to increase Ab-dependent cellular cytotoxicity and complement-dependent lysis of Env-expressing target cells through augmentation of Env-specific IgG Ab levels. These data indicate that the plasmid-delivered IL-21 and IL-15 can increase the magnitude of the response to DNA vaccines. ***The Journal of Immunology,*** **2006, 177: 177–191.**

The current challenge for the design of an effective HIV-1 vaccine is to develop immunization strategies to elicit both broader and sustain immune responses against diverse viral species. Such vaccines include those in which protective immune responses are stimulated by live, recombinant viruses, or DNA-based vectors (1–7), and augmented by a variety of cytokines or costimulatory molecules (8–16). Among cytokines that are of particular interest for the generation of an efficacious HIV-1 vaccine are those that share the common γ receptor chain (γ_c)[4] subunit and are implicated in the process of memory cell generation (17–19). Among them, IL-2, IL-4, IL-7, IL-15, and IL-21 are all involved in T cell responses and some of these cytokines are required for the development or maintenance of memory T cells (18, 20–24). IL-2, one of the most extensively studied cytokine that is produced by CD4$^+$ T cells, drives rapid clonal expansion of naive T cells leading to their subsequent differentiated state (18), but can also limit clonal expansion and the accumulation of Ag-specific effector cells by promoting activation-induced cell death (25). IL-7 is crucial for the survival and homeostatic expansion of naive CD8$^+$ T cells and can also contribute to memory CD8$^+$ T cell homeostasis (22, 26–28). IL-15, another survival factor, exerts its effect primarily on memory CD8$^+$ T cells (29, 30) by stimulating their proliferation rather than apoptosis and promoting memory T cell turnover (21, 24, 31–33). IL-15 can also provide a costimulus to induce B cell proliferation and Ig secretion (34). In the context of immune therapy and genetic vaccine, IL-15 has been shown to be effective against tumors as well as some infectious disease models (13, 35–39).

IL-21, the last member of the IL-2 cytokine family that exhibits functional overlaps with IL-2 and IL-15, has been suggested as an effective adjuvant in enhancing and sustaining CD8$^+$ T cell responses (40, 41). It efficiently promotes proliferation, cytotoxic activity, and IFN-γ production by murine and human CD8$^+$ effector T cells (41, 42). IL-21 also induces activated NK cell terminal differentiation and functions, thereby limiting NK cell expansion by IL-15 (43). In addition, a severe defect in IgG1 production following Ag priming in IL-21R-deficient mice (44),

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[4] Abbreviations used in this paper: γ_c, common γ receptor chain; ADCC, Ab-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; Env, envelope; RLU, relative light unit; rVV, recombinant vaccinia virus.

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indicates a role for IL-21 in regulating Ab production (44–46). In view of these findings, it has been suggested that IL-21 can influence both innate and adaptive immunity (43, 45). In fact, IL-21 has recently been shown to increase survival rates of HSV-1-infected mice when coinjected with a plasmid DNA encoding HSV-1 glycoprotein B Ag (47), and mice treated with IL-21 have been able to reject tumor cells and develop tumor-specific immune responses (40, 48–50).

Considering the dominant role that $CD4^+$ T cells and IL-2 cytokine family play in the generation and maintenance of HIV-1-specific immune responses, it seems important to better characterize the role of IL-21 in regulating the size of naive and memory T cell pools in response to HIV-1 Ags in vivo. This information is likely to facilitate the development of a rational approach for improving the efficacy of potential HIV-1 vaccines. In this study, we have investigated the ability of a plasmid-encoded IL-21 delivered alone or in combination with the *IL-15* gene to regulate the survival of envelope (Env)-specific $CD8^+$ T cell memory pool as well as the level and isotypes of Ab responses induced by the $gp140\Delta CFI_{HXB2/89.6}$ DNA expression vector in mice. We report here that IL-21 plasmid in a synergistic context with *IL-15* gene augmented expansion of the vaccine-induced memory $CD8^+$ T cells and mediated more vigorous recall responses to the vBD3 vaccinia virus challenge than animals immunized in the presence of either cytokine alone. The synergistic combination of IL-21 and IL-15 also promoted expansion of $CD8^+CD127^+$ memory T cell pools specific for the subdominant HLA-A2-restricted $Env_{121–129}$ epitope (KLTPLCVTL) (2), and has resulted in enhanced $CD8^+$ T cell function that was partially independent of $CD4^+$ T cell help in mediating protection against vBD3 challenge. The interaction between these cytokines also increased Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against Env-expressing target cells predominantly through augmentation of Env-specific IgG Ab levels.

Materials and Methods

Construction of the HIV-1 chimeric HxB2/89.6 Env DNA vaccine

The codon-optimized $gp140\Delta CFI_{HxB2}$ Env glycoprotein cloned in the VRC2801 expression vector (provided by Dr. G. Nabel, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) was used for generation of the HIV-1 chimeric $gp140\Delta CFI_{HxB2/89.6}$ construct (4). Briefly, $gp140\Delta CFI_{HxB2}$ was first subcloned to the pBluescript II SK^+ vector using *Bam*HI and *Xba*I restriction enzymes sites. Subsequently, two sites *Nsi*I and *Bsu*36I were introduced at positions 105 and 363, respectively, using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's procedure. After DNA sequence verification, the plasmid was digested with *Nsi*I and *Bsu*36I restriction enzymes and the entire fragment from residues 105 to 363 in $gp140\Delta CFI_{HxB2}$ was replaced with the corresponding sequence from 89.6 Env (accession number U39362) to create the Env chimeric construct. Then, the entire fragment between *Bam*HI and *Xba*I restriction sites was cloned into the VRC2801 expression vector. After the second DNA sequence verification, the expression of the $gp140\Delta CFI_{HxB2/89.6}$ gene was confirmed by immunoprecipitation of the Env glycoprotein from culture supernatants of radiolabeled 293T cell transfectants.

Transfection, labeling, and immunoprecipitation

Transient transfection of 293T cells with plasmid DNA encoding either the $gp140\Delta CFI_{HxB2}$ or $gp140\Delta CFI_{HxB2/89.6}$ gene was performed using Lipofectamine Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. For the radioimmunoprecipitation analysis, [^{35}S]methionine/cysteine (400 μCi/plate; DuPont-New England Nuclear) was added on the second day of transfection for an additional 24-h period. At the end of the incubation period, culture supernatants were collected, concentrated 100-fold on a Millipore filter (Millipore), and immunoprecipitated with a mixture of sera from HIV-1-infected individuals (51) followed by polyclonal rabbit anti-human Ig (ICN Biomedicals) and protein A-Sepharose CL-4B (Pharmacia Biotech). The immunoprecipitates were separated by Blue Native PAGE (BN-PAGE) as described (52). Typically, 5 μg of purified proteins was loaded per lane, and the gels were fluorographed to visualize [^{35}S]methionine/cysteine-labeled proteins.

Immunization of rabbits

Female New Zealand rabbits were immunized with the Env vaccine at the Lampire Biologicals. Briefly, each rabbit received two i.m. $gp140\Delta CFI_{HxB2/89.6}$ DNA injections (500 μg per rabbit) in a 3-wk interval followed by three protein boosts. For the booster immunization, soluble $gp140\Delta CFI_{HxB2/89.6}$ Env glycoprotein was purified from culture supernatants of 293T transfectants by lectin affinity chromatography, emulsified with IFA, and injected (500 μg per rabbit) s.c. on days 42, 63, and 84. Before immunization, the level and integrity of the purified Env were confirmed by the BN-PAGE analysis. The immune sera were collected on day 94 and checked for reactivity with the Env glycoprotein by an ELISA. Preimmunized sera were included as negative controls.

Env-pseudotyped virus neutralization and luciferase reporter cell assay

The assay was performed as previously described (53, 54). Briefly, a HeLa cell line that expresses CD4, CXCR4, and CCR5 was used as target cells for HIV-1 infection. These cells, initially called JC53-bl and now termed TZM-bl, were obtained from the National Institutes of Health AIDS Reference and Reagent Repository (Germantown, MD). The cells contain Tat-responsive reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under regulatory control of an HIV-1 long-terminal repeat. In the assay, the level of HIV-1 infection was quantified by measuring relative light units (RLUs) of luminescence that are directly proportional to the amount of virus input.

One day before the neutralization assay setup, 3000 TZM-bl cells were plated in a 96-well flat-bottom tissue culture plate in 100 μl of complete medium. Dilutions of heat-inactivated (56°C for 1 h) sera from pre- and postimmunized rabbits were made in duplicate in 96-well round-bottom tissue culture plates. A predetermined amount of pseudovirus that resulted in 2 to 5×10^5 RLUs was added to the diluted sera for 1.5 h at 37°C. In separate wells, cells were incubated with virus in the absence of the Abs. During the incubation, the TZM-bl cells were treated with polybrene (2 μg/ml) for 30 min at 37°C. The medium containing polybrene was aspirated from the cells and replaced with 50 μl of fresh medium combined with 50 μl of the virus/serum mixture and incubated for 3 days at 37°C. Cell lysis occurred by replacing the medium with 100 μl of 1× Cell Culture Lysis Reagent (Promega). Cells were lysed for 1 h at room temperature and subjected to one freeze-thaw cycle. Then 60 μl of the lysate was loaded onto a corresponding well in a Black Enhanced Binding 96-well solid plate, a luciferase substrate (Promega) was added to each well (100 μl /well), and the RLUs associated with each well were recorded with a Fluoroskan Ascent FL (Thermo Labsystems). Percent neutralizations were calculated as previously described (54).

Recombinant vaccinia viruses (rVVs)

The rVVs expressing the full-length Env of $HIV\text{-}1_{89.6}$ (vBD3) (55) and β-galactosidase (vSC8) (56) were provided by Drs. R. Collman (University of Pennsylvania, Philadelphia, PA) and B. Moss (Laboratories of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), respectively.

Mice and immunization

Six-week-old BALB/c ($H\text{-}2^d$) mice were purchased from The Jackson Laboratory. The HLA-A2/K^b-transgenic mice, expressing hybrid molecules bearing the α1 and α2 domains of HLA-A2.1 fused to the α3 domain of $H\text{-}2K^b$, were provided by Dr. L. Sherman (The Scripps Research Institute, San Diego, CA). All mice were maintained in a specific pathogen-free microisolator environment, and the experimental procedures were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Roswell Park Cancer Institute. Mice were injected i.m. in both quadriceps with the $gp140\Delta CFI_{HxB2/89.6}$ vector (10, 30, 100, or 300 μg/mouse). The immunization was repeated three times in a 3-wk interval. Control mice were immunized with the sham plasmid. The IL-21- or IL-15-encoded pORF9 vector (10, 20, or 40 μg/mouse; InvivoGen) was delivered i.m. at the time of vaccination with the $gp140\Delta CFI_{HxB2/89.6}$ construct. Two additional groups of $gp140\Delta CFI_{HxB2/89.6}$-immunized mice received IL-21 plasmid on day 5 or 15 after vaccination. The optimal dose of the IL-21 and IL-15 expression vectors that were used in combination with the $gp140\Delta CFI_{HxB2/89.6}$ vaccine was determined using the vBD3 vaccinia virus challenge study.

Protection against challenge with the vBD3 vaccinia virus

At different time points after the last DNA immunization, mice were challenged i.p. with 2×10^7 PFU of vBD3 or vSC8 rVV. Five days later, mice were sacrificed, ovaries removed, homogenized, sonicated, and assayed for vaccinia virus titer by plating serial 10-fold dilutions on human HuTK$^-$ 143B indicator cells, staining with crystal violet and counting plaques at each dilution as described (2). To evaluate the contribution of CD8$^+$ and CD4$^+$ T cells as well as NK cells to the vaccine-induced protection against the vBD3 challenge, gp140ΔCFI$_{HxB2/89.6}$-immunized mice were treated i.p. with 100 μg of anti-CD4 mAb (clone GK 1.5), anti-CD8 mAb (clone 53-6.72), or with 25 μl of anti-asialo GM1 (GA1) rabbit serum (Wako Chemicals) on days 3 and 1 before and day 1 after the challenge as described (57, 58). To investigate whether IL-15 and IL-21 would enhance the gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced protection against vBD3 challenge in mice that were partially depleted of CD4$^+$ or CD8$^+$ T cells, BALB/c mice were injected i.p. with anti-CD4 or anti-CD8 mAb before and during the immunization period. The Ab treatment was capable of depleting CD4$^+$, CD8$^+$, or NK cells in nonimmunized mice by 90% as determined by a flow cytometric analysis with anti-CD4 (clone H129.19), anti-CD8 (clone 53-5.8), and CD49b/Pan-NK cells (clone DX5) mAbs (BD Pharmingen). In parallel, control groups of gp140ΔCFI$_{HxB2/89.6}$-immunized mice were treated with 100 μg of rat IgG (ICN Biomedicals).

Cytotoxicity assay

Splenocytes from BALB/c mice were cultured at 2×10^6 cells/ml in 24-well culture plates with vBD3-infected monocyte-macrophage cell line PU5–1.8 (American Type Culture Collection) and 15% T cell stimulatory factor (T-STIM Culture Supplement; Collaborative Biomedical Products) as a source of exogenous IL-2. Before incubation, vBD3-infected stimulator cells were incubated with 0.5% paraformaldehyde on ice to inactivate the virus (51), washed, and mixed with splenocytes at a 1:40 ratio. After 3 days of stimulation, cells were split and cultured in medium supplemented with 0.3 ng/ml recombinant mouse IL-2 (BD Pharmingen). The cytolytic activity was analyzed 6 days later by a standard 4-h ^{51}Cr release assay against P815 cells infected with vBD3. Control target cells were infected with vSC8. The percent specific lysis was calculated as: ((cpm experimental release $-$ cpm spontaneous release)/(cpm maximum release $-$ cpm spontaneous release)) $\times$ 100. Maximum release was determined from supernatants of cells that were lysed by addition of 5% Triton X-100. Spontaneous release was determined from target cells incubated with medium only.

ELISPOT assay

The numbers of Env-specific and IFN-γ-secreting CD8$^+$ splenocytes in gp140ΔCFI$_{HxB2/89.6}$-immunized and control mice were determined by the ELISPOT assay as described (59). Briefly, 96-well nitrocellulose plates (Millipore Multiscreen-MAIP; Millipore) were coated with 15 μg/ml rat anti-mouse mAb directed to IFN-γ (MAB785; R&D Systems) in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the wells were washed with PBS containing 0.05% Tween 20 and blocked for 1 h with RPMI 1640 medium containing 10% FCS. For the analysis of frequencies of Env-specific and IFN-γ-secreting CD8$^+$ T cells, splenocytes were combined with vBD3-infected P815 cells at a ratio of 3:1, and placed in 2-fold dilutions into the Ab-coated wells. For each dilution, duplicate samples were used. After 24 h of incubation at 37°C, the plates were washed six times with PBS containing 0.05% Tween 20 and incubated for 2 h with 50 μl of 1 μg/ml biotinylated mAb directed to mouse IFN-γ (MAB485; R&D Systems). The plates were washed and incubated for 1 h with 50 μl of 1/1000 diluted streptavidin-conjugated alkaline phosphatase (SA-5100; Vector Laboratories). After final wash with PBS, spots were developed with an alkaline phosphatase 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP)/NBT substrate (SK-5400; Vector Laboratories) and counted under a stereomicroscope. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots vs the number of effector cells.

HLA-tetramer analysis

MHC class I tetramers folded with the subdominant HLA-A*0201-restricted Env$_{121–129}$ epitope (KLTPLCVTL) (2) was prepared as previously described (60). Splenocytes harvested from gp140ΔCFI$_{HxB2/89.6}$-immunized HLA-2/K^b-transgenic mice were incubated with Fc Block (anti-CD16/32) for 10 min and then labeled with PE-Env$_{121–129}$/HLA-A*0201 tetramer and anti-CD8α-CyChrome mAb in combination with either anti-CD44-FITC, anti-CD62L-FITC, or anti-CD127 (IL-7Rα)-FITC mAb (BD Pharmingen). The cells were washed twice in HBSS containing 0.01% NaN_3 and 1% FCS, fixed with 1% paraformaldehyde, and stored at 4°C in the dark before acquisition. Background staining was assessed using an isotype control Ab. The number of Env$_{121–129}$/HLA-A*0201 tetramer-specific CD8$^+$ T cells secreting IFN-γ was determined using BD Cytofix/Cytosperm kit and allophycocyanin-conjugated IFN-γ mAb (BD Pharmingen) according to the manufacturer's protocol. All flow cytometric evaluations were performed on FACScan or FACSCalibur flow cytometer. After gating on forward and side scatter parameters, at least 10,000-gated events were routinely acquired and analyzed using CellQuest software (BD Biosciences).

Measurement of Env-specific Ab end point titers

To detect serum Abs against the Env glycoprotein by Con A ELISA, plates were coated with 100 μl of *Galanthus nivalis* lectin (Sigma-Aldrich) (10 μg/ml) overnight at 4°C. The lectin solution was removed from the wells and blocked with 200 μl of PBS containing 10% FBS for 2 h at room temperature. The plates were washed twice with PBS containing 0.2% Tween 20 (PBST), and then coated with the Env glycoprotein purified on a lectin-column from supernatant of vBD3-infected 293T cells. The plates were washed with PBST five times, and then sera from different groups of the immunized and cytokine-treated mice were added in 3-fold dilutions. After a 1-h incubation at room temperature, the plates were washed five times and incubated with a 1/1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM or IgG (Sigma-Aldrich) in PBST. Subisotyping of serum IgG Ab responses in the immunized mice was conducted with alkaline phosphatase-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, or IgG3 secondary Abs (BD Pharmingen). After washing, the reaction was developed with *p*-nitrophenylphosphate (1 mg/ml; Bio-Rad) in diethanolamine buffer (Bio-Rad), stopped with 0.4 M NaOH, and analyzed at 405 nm with an ELISA plate reader Dynatech MRX. Sample dilutions were considered positive if the OD recorded for that dilution was at least 2-fold higher than the OD recorded for the control sample at the same dilution (61).

ADCC and CDC assays

For the ADCC assay, different dilutions of sera from the immunized mice were incubated for 6 h with ^{51}Cr-labeled P815 cells infected with vBD3 or vSC8 vaccinia virus together with NK cell-enriched splenocytes isolated from naive BALB/c mice. NK cells were enriched by centrifugation over a discontinuous density gradient consisting of 70, 65, 60, 57, 55, and 50% Percoll (Amersham Biosciences) as described (62), and recovered in the lower density fractions (63).

The CDC against cells expressing the Env glycoprotein was assayed at serum dilutions of 1/30, 1/90, and 1/270 with rabbit complement (Cedarlane Laboratories) by a standard ^{51}Cr release assay against vBD3-infected P815 cells as described (64). Cells incubated only with medium, complement, or antisera served as controls. Spontaneous release was calculated based on the chromium released by target cells incubated with complement alone. Maximum radioactivity release was determined from supernatants of cells that were lysed by the addition of 5% Triton X-100. The percent of specific lysis was calculated as: ((cpm experimental release $-$ cpm spontaneous release)/(cpm maximum release $-$ cpm spontaneous release)) $\times$ 100.

Statistical analyses

The significance of differences in the number of Env-specific CD8$^+$ T cells and Env-specific Ab titers in different groups of the immunized mice was determined using a two-tailed Student's *t* test assuming equal variance. Mixed-model ANOVA (65) was used to compare mean values of the vBD3 viral titers between control mice and those immunized with gp140ΔCFI$_{HxB2/89.6}$ in the presence or absence of IL-15 and IL-21 plasmids. The *p* values for the pairwise group comparisons for the average vBD3 virus titers in the immunized mice that were challenged over the 6-mo period were computed using the nonparametric Wilcoxon's rank-sum test. Data were presented as arithmetic mean $\pm$ SD and analyzed using the JMP software (SAS Institute) on a Windows-based platform.

Results

Expression of the gp140ΔCFI$_{HxB2/89.6}$ mutant and induction of neutralizing Ab responses

For the DNA vaccination study, we have expressed the CCR5-tropic version of the gp140ΔCFI$_{HxB2/89.6}$ Env glycoprotein on the backbone the codon-optimized gp140ΔCFI$_{HXB2}$ construct, which has been previously modified to reduce gp160-induced cytotoxicity, stabilize the formation of trimers, and eliminate formation of the hairpin intermediate (4). In the gp140ΔCFI$_{HxB2/89.6}$ mutant,

the most divergent region encoding aa 105–363 in gp140ΔCFI$_{HXB2}$ was replaced with the corresponding sequence from the 89.6 Env glycoprotein (Fig. 1*A*). After DNA sequence verification, the oligomeric state of the secreted gp140ΔCFI$_{HxB2/89.6}$ Env glycoprotein in culture supernatants of transiently transfected 293T cells was analyzed by immunoprecipitation and BN-PAGE. Supernatants from cells transfected with the gp140ΔCFI$_{HXB2}$ construct (Fig. 1*B*, *lane 2*) and the sham plasmid (Fig. 1*B*, *lane 1*) served as positive and negative controls, respectively. Consistent with the described patterns for the gp140ΔCFI$_{HXB2}$ Env glycoprotein expression (4), the gp140ΔCFI$_{HxB2/89.6}$ mutant predominantly formed trimeric and dimeric oligomers (Fig. 1*B*, *lane 3*). Notably, there was a significant component of trimers that migrated as a well-resolved band with a molecular mass of ~420 kDa, showing that the gp140ΔCFI$_{HxB2/89.6}$ Env is capable of forming the appropriate oligomer. Typical of such analyses (66), some aggregation has also occurred based on the presence of additional high-molecular-mass (>500-kDa) multimers. Despite a similar pattern of oligomerization, the gp140ΔCFI$_{HxB2/89.6}$ and gp140ΔCFI$_{HXB2}$ glycoproteins could be distinguished from each other based on a lower electrophoretic mobility of the gp140ΔCFI$_{HxB2/89.6}$ mutant due to the presence of two additional *N*-glycosylation sites, which increased its molecular mass by ~5 kDa (67).

The ability of the gp140ΔCFI$_{HxB2/89.6}$ glycoprotein to elicit neutralizing Ab responses against primary HIV-1 isolates was determined in rabbits because of a high background generated with murine sera. Rabbits also produce Abs with long HCDR3 finger-like structures that have been linked to being important in the broadly neutralizing human mAbs b12, 2F5, and 4E10 (68), whereas mice produce such Abs very rarely. Three rabbits were immunized twice with the gp140ΔCFI$_{HxB2/89.6}$ plasmid DNA followed by three booster immunizations with the recombinant gp140ΔCFI$_{HxB2/89.6}$ glycoprotein emulsified with IFA. In all cases, the Env-specific Ab production was increased from undetectable levels, in preimmune animals, to the titers ranging from 1.5×10^5 to 3.0×10^5 after the last booster immunization. This is in contrast with the less consistent and lower titers of Ab responses elicited by the previously reported gp140$_{89.6}$ oligomeric Env generated as a gp120-gp41 cleavage site mutant wherein the furin cleavage site was replaced with a hexameric Leu-Arg motif (69).

The neutralizing Ab responses elicited by the gp140ΔCFI$_{HxB2/89.6}$ vaccine were examined against a panel of clade B HIV-1 isolates, including 89.6, SF162, HxB2, TORNO, QH0692, and SS1196, (70). The preimmune and immune rabbit sera were diluted 10-fold and analyzed for neutralizing activities in Env-pseudotyped virus neutralization and luciferase reporter cell assay.

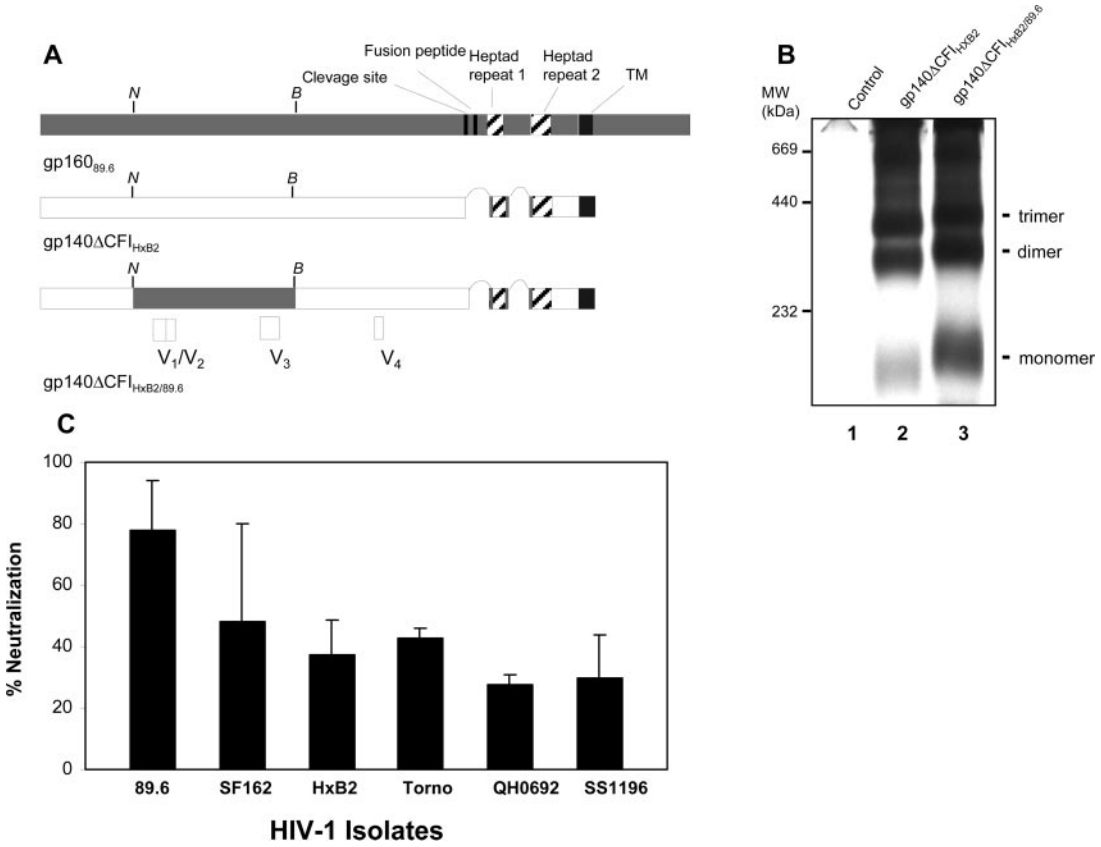

FIGURE 1. Expression of the gp140ΔCFI$_{HxB2/89.6}$ Env glycoprotein and induction of neutralizing Ab responses. *A*, Schematic representation of the gp140ΔCFI$_{HxB2/89.6}$ expression construct. In the HIV-1 gp140ΔCFI$_{HxB2/89.6}$ chimeric vector, the region from residues 105 to 363 in gp140ΔCFI$_{HXB2}$ was replaced with the corresponding sequence from the HIV-1$_{89.6}$ Env glycoprotein (in gray). Abbreviations for the restriction enzyme sites *Nsi*I and *Bsu*36I are denoted as *N* and *B*, respectively. *B*, Expression of gp140ΔCFI$_{HxB2/89.6}$ and gp140ΔCFI$_{HXB2}$ glycoproteins in culture supernatants from 293T transfectants. Control cells were transfected with the backbone plasmid (*lane 1*). The oligomeric states of secreted gp140ΔCFI$_{HXB2}$ and gp140ΔCFI$_{HxB2/89.6}$ glycoproteins were confirmed by BN-PAGE on 4–12% Bis-Tris NuPAGE gel (*lanes 2* and *3*, respectively). *C*, Generation of neutralizing Ab response by the gp140ΔCFI$_{HxB2/89.6}$ vaccine in rabbits. Sera were collected from three rabbits before immunization and after the prime/boost immunization with the gp140ΔCFI$_{HXB2}$ DNA construct and recombinant protein as described in *Materials and Methods*. Both preimmune and postimmune sera were tested at a 1/10 dilution for neutralizing activity against a panel of HIV-1 isolates. Neutralizing Ab titers were analyzed as previously described (54). SDs are indicated.

Fig. 1*C* shows that at the 1/10 serum dilution, the gp140ΔCFI$_{HxB2/89.6}$ construct-immunized rabbit sera had a mean (± SD) neutralization activity of 78 ± 16% against the homologous 89.6 virus. Other HIV-1 isolates were neutralized less effectively with the activities ranging from 27.5 to 48%. Collectively, these results demonstrated that the gp140ΔCFI$_{HxB2/89.6}$ vaccine is capable of inducing a neutralizing response to functional Env glycoproteins on homologous and, to a lesser extent, some heterologous HIV-1 isolates.

IL-21 augments gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced protection against challenge with vBD3 vaccinia virus

Using a challenge model with Env$_{89.6}$-expressing recombinant vBD3 vaccinia virus as a surrogate for HIV-1 infection (2), we examined the ability of the gp140ΔCFI$_{HxB2/89.6}$ vaccine to induce protective cellular responses in mice. Because the vaccinia virus does not incorporate the Env glycoprotein in the virion but expresses it only in the infected cells, the observed protection is predominantly mediated by the vaccine-induced CTLs rather than Ab responses. Groups of BALB/c mice ($n = 5$) were injected with 10, 30, 100, or 300 μg of gp140ΔCFI$_{HxB2/89.6}$ vector in both hind legs in the quadriceps muscle. The immunization was repeated every 3 wk, and the vaccinated mice were challenged with vBD3 or vSC8 virus 5 wk after the third immunization. The latter vaccinia virus expressing β-galactosidase served as a specificity control. Because vaccinia virus replicates most efficiently in ovaries (71), ovaries were removed 5 days after the challenge and tested for viral titers on a monolayer of HuTK$^-$143 cell line. As shown in Fig. 2*A*, the vaccinia titers in ovaries of mice immunized with 10 or 30 μg of the gp140ΔCFI$_{HxB2/89.6}$ construct and challenged with vBD3 were ~10-fold lower than in vSC8-challenged mice or in animals immunized with the sham plasmid. The highest level of protection was detected in mice immunized with 100 or 300 μg of the gp140ΔCFI$_{HxB2/89.6}$ plasmid ($p < 0.05$). In these mice, the viral load was ~100-fold lower compared with the vSC8-challenged counterparts. Similar results were obtained by Chakrabarti et al. (4) using 100 μg of the gp140ΔCFI$_{HXB2}$ plasmid DNA for induction of optimal Env-specific immune responses in mice.

The effect of IL-21 on the level of gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced immunity was first examined in a challenge study in BALB/c mice. In these experiments, mice were immunized three times with 100 μg of the gp140ΔCFI$_{HxB2/89.6}$ construct delivered alone or in combination with 10, 20, or 40 μg of IL-21 expression vector. The challenge with either vBD3 or vSC8 virus was conducted 5 wk after the last immunization, and the vaccinia titers were evaluated in the ovaries 5 days later. Fig. 2*B* shows that 20 and 40 μg of IL-21 plasmid had the most impact on protection by lowering the vaccinia titer by ~3 $\log_{10}$ compared with vSC8-challenged mice ($p < 0.01$), whereas 10 μg of the plasmid was less effective in decreasing the vBD3 titer in the immunized animals.

We next used the vBD3 challenge approach to determine the effect of IL-21 delivery relative to the DNA vaccination on the level and duration of gp140ΔCFI$_{HxB2/89.6}$-induced immune responses. The plasmid-encoded IL-21 (20 μg/mouse) was injected either simultaneously with the gp140ΔCFI$_{HxB2/89.6}$ construct (100 μg/mouse) or 5 and 15 days after specific vaccination. Two additional booster immunizations with the gp140ΔCFI$_{HxB2/89.6}$ construct alone or in combination with IL-21 vector were conducted every 3 wk. The level of Env-specific immune responses in the immunized and IL-21 plasmid-treated mice were monitored every

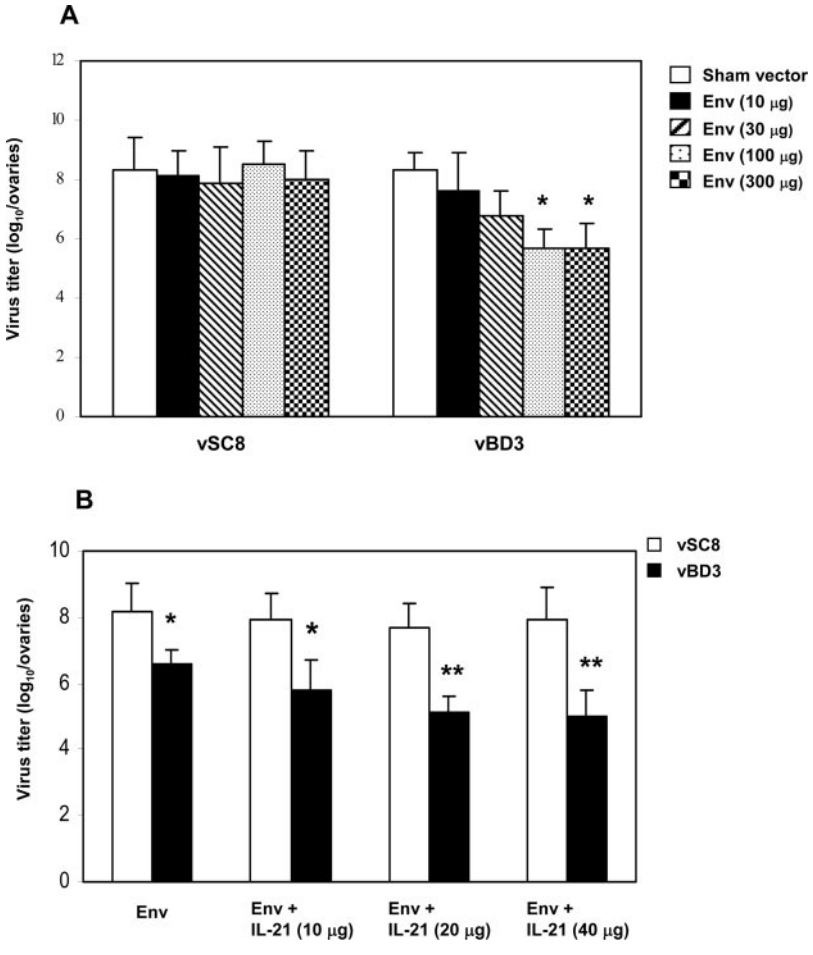

FIGURE 2. Protection against challenge with vBD3 vaccinia virus following immunization with the gp140ΔCFI$_{HxB2/89.6}$ construct alone or in combination with a plasmid-encoded IL-21. *A*, BALB/c mice were immunized i.m. with DNA without an insert (sham vector) or a vector expressing gp140ΔCFI$_{HxB2/89.6}$ Env (10, 30, 100, or 300 μg/mouse). Five weeks after the third immunization, the mice were challenged i.p. with 2×10^7 PFU of vBD3 or vSC8. Five days later, the mice were sacrificed, ovaries removed, homogenized, and assayed for vaccinia virus titers by plating serial 10-fold dilutions on HuTK$^-$143 cells, staining with crystal violet, and counting plaques at each dilution. *B*, BALB/c mice were immunized with the gp140ΔCFI$_{HxB2/89.6}$ construct alone or in combination with a plasmid-encoded IL-21 (10, 20, or 40 μg/mouse) before a challenge with vBD3 or vSC8 virus. The vaccinia titers are presented as the mean $\log_{10}$ ± SD (error bars) of PFU per ovaries of five mice per group. *, $p < 0.05$ and **, $p < 0.01$.

2 mo by measuring protection against vBD3 challenge. To determine whether any protection was Env-specific, additional groups of the immunized mice were challenged with vSC8 vaccinia virus. Fig. 3 shows that administration of IL-21 vector enhanced to a varying degree the overall protection level against vBD3 challenge all mice immunized with the gp140ΔCFI$_{HxB2/89.6}$ vaccine. Consistent with the notion that IL-21 exposure to an immune system that has been primed with a specific Ag may lead to the optimal augmentation of immune responses (40), administration of IL-21 vector 5 days after gp140ΔCFI$_{HxB2/89.6}$ vaccination elicited the highest attenuation of vBD3 replication that lasted for 6 mo after the last booster immunization (Fig. 3). The protection against vBD3 in mice that received IL-21 together with the gp140ΔCFI$_{HxB2/89.6}$ vaccine or 15 days after immunization lasted for ~3 mo, and was higher compared with that elicited by immunization with the gp140ΔCFI$_{HxB2/89.6}$ vector only (Fig. 3, *A* and *B*). The observed protection against challenge with vBD3 was specific as no inhibition of vSC8 replication was detected in the same groups of the immunized mice.

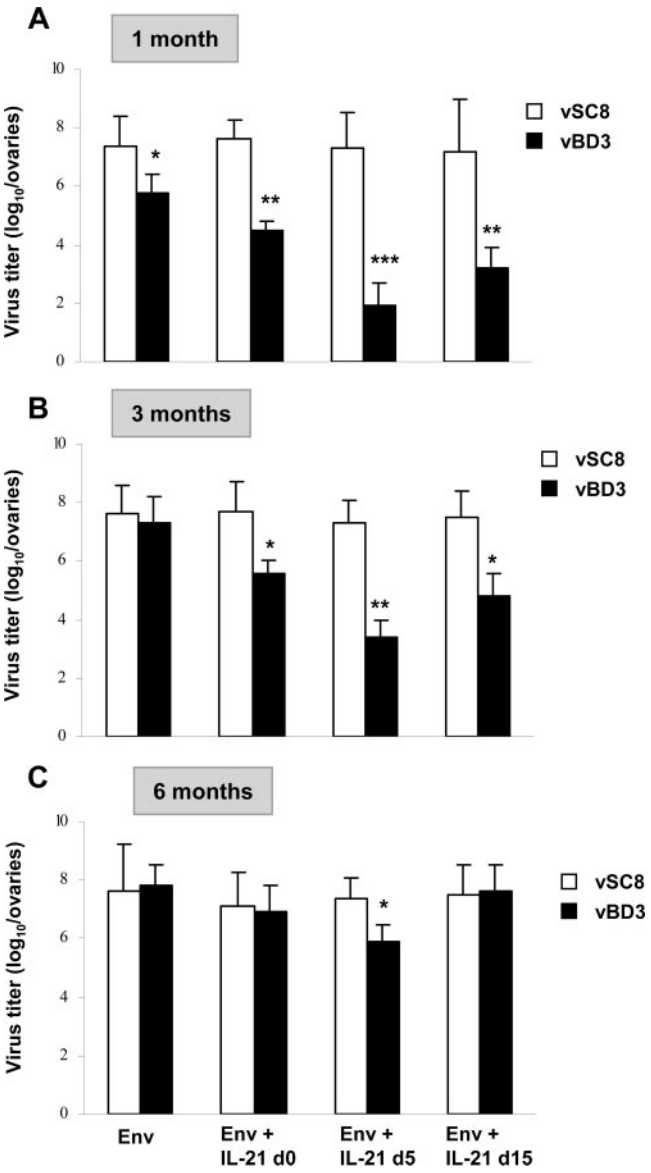

FIGURE 3. Effect of IL-21 delivery relative to the gp140ΔCFI$_{HxB2/89.6}$ DNA immunization on the level and duration of the Env vaccine-induced protection against challenge with vBD3 virus. *A*, BALB/c mice were immunized three times with the gp140ΔCFI$_{HxB2/89.6}$ construct alone or in combination with IL-21. The IL-21 plasmid was injected simultaneously with the Env vaccine (day 0), or 5 and 15 days later. The immunized mice were challenged with 2×10^7 PFU of vBD3 (■) and vSC8 (□) 1 mo after the third immunization. The viral titers in ovaries were analyzed 5 days after the challenge and tested for vaccinia titers on a monolayer of HuTK$^-$143 cell line. *B*, The titers of vBD3 and vSC8 in ovaries of gp140ΔCFI$_{HxB2/89.6}$-immunized and IL-21-treated mice were analyzed 3 mo after vaccination. *C*, The titers of vBD3 and vSC8 in ovaries of gp140ΔCFI$_{HxB2/89.6}$-immunized and IL-21-treated mice were analyzed 6 mo after vaccination. The values are presented as the mean $\log_{10} \pm$ SD (error bars) of PFU per ovaries of five mice per group. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

IL-21 acts synergistically with IL-15 to enhance gp140ΔCFI$_{HxB2/89.6}$-mediated protection

IL-21 has been reported to synergize with IL-15 in boosting Ag-specific CD8$^+$ T cell expansion ex vivo, and during an adoptive transfer of B16 melanoma in sublethally irradiated mice (41). To further explore the actions of IL-21 and test its ability to synergize with IL-15 in enhancing DNA vaccine-induced immune responses, we examined to what extent IL-21 expression vector alters the immune response to the Env glycoprotein in gp140ΔCFI$_{HxB2/89.6}$-immunized and IL-15-treated BALB/c mice. The optimal dose of the IL-15 expression vector (20 μg/mouse), determined by the vBD3 challenge study (data not shown), was administered together with the gp140ΔCFI$_{HxB2/89.6}$ construct as previously described for the Env DNA vaccine (8), whereas IL-21 plasmid was delivered 5 days after the specific immunization. All mice were challenged i.p. with vBD3 or vSC8 vaccinia virus 5 wk after the third vaccination and analyzed for the protection level.

Consistent with results of the previous experiments, the vBD3 titer in ovaries of gp140ΔCFI$_{HxB2/89.6}$-immunized mice was ~100-fold lower compared with vSC8-challenged animals (Fig. 4*A*). Administration of *IL-15* or *IL-21* gene had a similar effect on the vaccine-induced protection reducing replication of vBD3 virus by ~5 $\log_{10}$ ($p < 0.001$). However, the combination of IL-15 and IL-21 plasmids was more potent than the effect of either cytokine alone on the gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced protection providing almost complete eradication of the virus. There were no significant differences between vSC8 titers in control mice and those immunized with the gp140ΔCFI$_{HxB2/89.6}$ vaccine and treated with the cytokine genes, indicating that the observed synergy of IL-21 and IL-15 in providing protection against the vBD3 challenge could not be mediated by nonspecific inflammatory responses.

IL-15 and IL-21 augment the effector function of Env-specific CD8$^+$ T cells responses

We next analyzed IL-15- and IL-21-induced changes in CD8$^+$ T cells responses elicited by the gp140ΔCFI$_{HxB2/89.6}$ vaccine in BALB/c mice by a standard 4-h ^{51}Cr release assay against vBD3-infected P815 mastocytoma cells expressing only the MHC class I (H-2^d) molecules (59). Target cells infected with vSC8 virus or splenocytes from sham plasmid-immunized mice were used as specificity controls. Consistent with the in vivo analyses, the highest level of Env-specific CTL activities was measured in mice coimmunized with the IL-15 and IL-21 expression vectors (Fig. 4*B*). In these animals, vBD3-infected cells were lyzed with the highest level over a broad range of E:T ratios. The gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced CTL activities in mice that received either IL-21 or IL-15 vector were at relatively comparable levels and ~2-fold higher than those in the untreated animals.

In parallel experiments, the effect of *IL-15* and *IL-21* genes on the frequency of IFN-γ-secreting CD8$^+$ splenocytes in

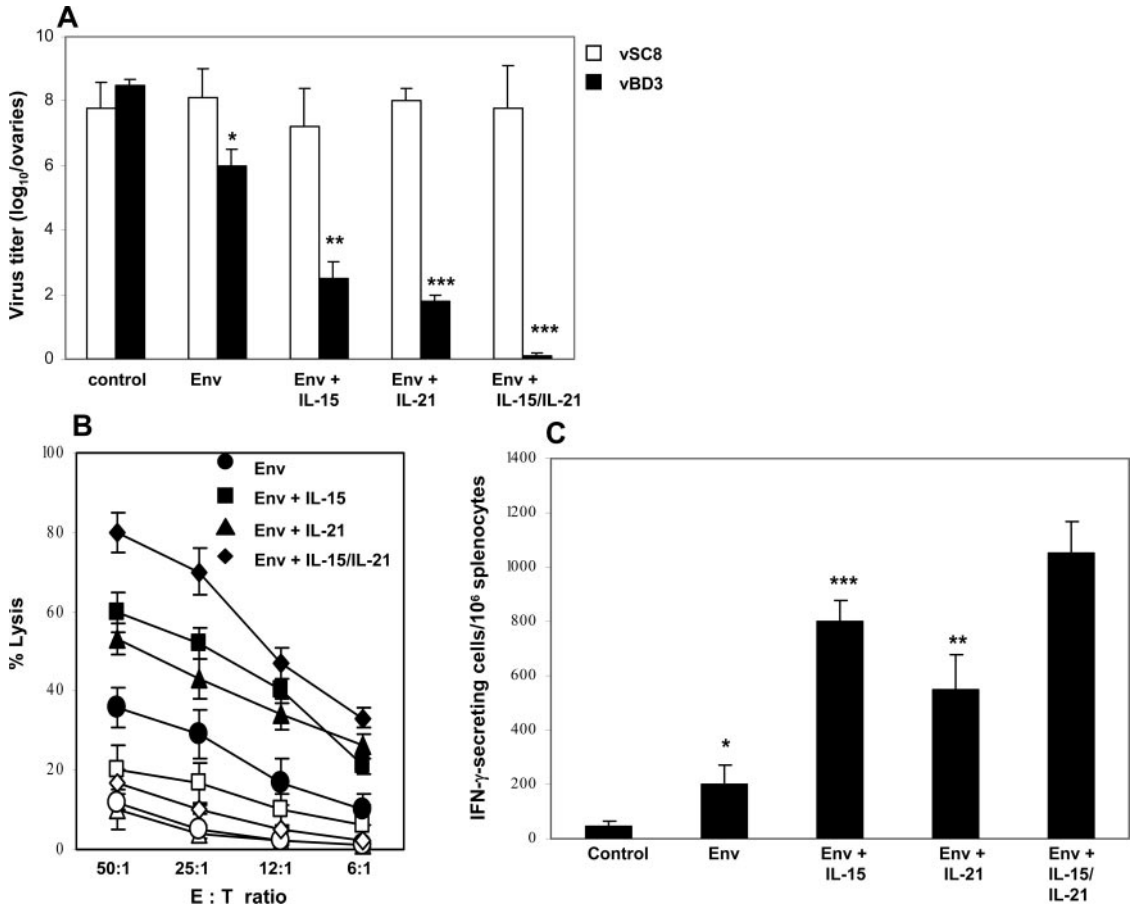

FIGURE 4. Effect of *IL-15* and *IL-21* genes on the Env vaccine-induced $CD8^+$ T cell responses. *A*, Protection against vBD3 challenge. Five weeks after the third immunization with the gp140ΔCFI$_{HxB2/89.6}$ construct in the presence, absence or combination of *IL-15* and *IL-21* genes, BALB/c mice were challenged i.p. with 2×10^7 PFU of vBD3 (■) or vSC8 (□). Control mice were immunized with the sham vector. Five days later, mice were sacrificed, ovaries removed, homogenized, and assayed for vaccinia virus titers. The titers are presented as the mean $\log_{10} \pm$ SD (error bars) of PFU per ovaries of five mice per group. *B*, Analysis of Env-specific CTL responses in mice immunized with the gp140ΔCFI$_{89.6}$ construct in the presence, absence or combination of IL-15 and IL-21 expression vectors. The Env-specific CTL response was analyzed in a standard ^{51}Cr release assay against P815 cells infected with vBD3 (solid symbols) or vSC8 (open symbols). All determinations were made in triplicate samples, and the SD was <10%. Results are presented as the means ± SD (error bars) of three independent experiments. *C*, The numbers of IFN-γ-secreting $CD8^+$ T cells in splenocyte cultures established from mice immunized with the gp140ΔCFI$_{HxB2/89.6}$ construct were determined by ELISPOT assay with vBD3-infected P815 cells. Control cells derived from mice immunized with the sham plasmid. Results are presented as the means ± SD (error bars) of at least three independent experiments. The frequencies of IFN-γ-secreting cells were determined by regression analysis from a curve generated by plotting the number of spots vs the number of effector cells. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

gp140ΔCFI$_{HxB2/89.6}$-immunized and control mice was analyzed by ELISPOT assay against vBD3-infected P815 cells. As shown in Fig. 4*C*, the IL-15 and IL-21 combination resulted in a marked increase in the number of IFN-γ-secreting $CD8^+$ T cells compared with that detected in gp140ΔCFI$_{HxB2/89.6}$-immunized mice ($p <$ 0.001). Mice that were treated with the plasmid-derived IL-15 at the time of specific vaccination also had elevated numbers of IFN-γ-secreting $CD8^+$ T cells, and higher than those elicited by the IL-21 treatment.

IL-15 and IL-21 enhance the expansion of $CD8^+$ T cells to a subdominant Env$_{121–129}$ epitope in gp140ΔCFI$_{HxB2/89.6}$-immunized HLA-A2/K^b mice

As new approaches for increasing the size and breadth of effector and memory pools induced after immunization are needed to improve the prospect of generating an effective HIV-1 vaccine, we investigated whether IL-15 and IL-21 cytokines would augment the vaccine-induced $CD8^+$ T cells to the subdominant Env$_{121–129}$ epitope (KLTPLCVTL) with HLA-A*0201-binding motif. To this end, splenocytes from the HLA-A2/K^b-transgenic mice that were immunized with the gp140ΔCFI$_{HxB2/89.6}$ vaccine in the presence or absence of IL-15 and IL-21 expression vectors were examined for Env$_{121–129}$/HLA-A*0201 tetramer$^+$ $CD8^+$ T cells by a flow cytometric analysis 5 wk after the third booster vaccination. As shown in Fig. 5, the frequency of tetramer-positive and $CD8^+$ T cells in the immunized mice that were treated with *IL-15* and *IL-21* genes ranged from 2.1×10^6 to 2.9×10^6 cells, and were ~2-fold higher compared with the untreated counterparts ($2.43 \pm 0.41 \times 10^6$ cells vs $1.26 \pm 0.22 \times 10^6$ cells, $p = 0.04$). In the latter group of mice, the number of tetramer-positive $CD8^+$ T cells ranged from 1.0×10^6 to 1.5×10^6 cells (Fig. 5*A*). To obtain a more comprehensive understanding of the effect of the plasmid-derived IL-15 and IL-21 on the memory cell development in gp140ΔCFI$_{HxB2/89.6}$-immunized mice, we also analyzed the cell surface expression CD44, CD62L, and CD127 on Env$_{121–129}$/HLA-A*0201 tetramer$^+$ $CD8^+$ T cells. In both groups of mice, the majority of tetramer-positive $CD8^+$ T cells expressed CD44 as well as CD62L and CD127 Ags (Fig. 5, *A* and *B*, *right panels*), suggesting that they could be selected to survive and develop into long-lived memory cells.

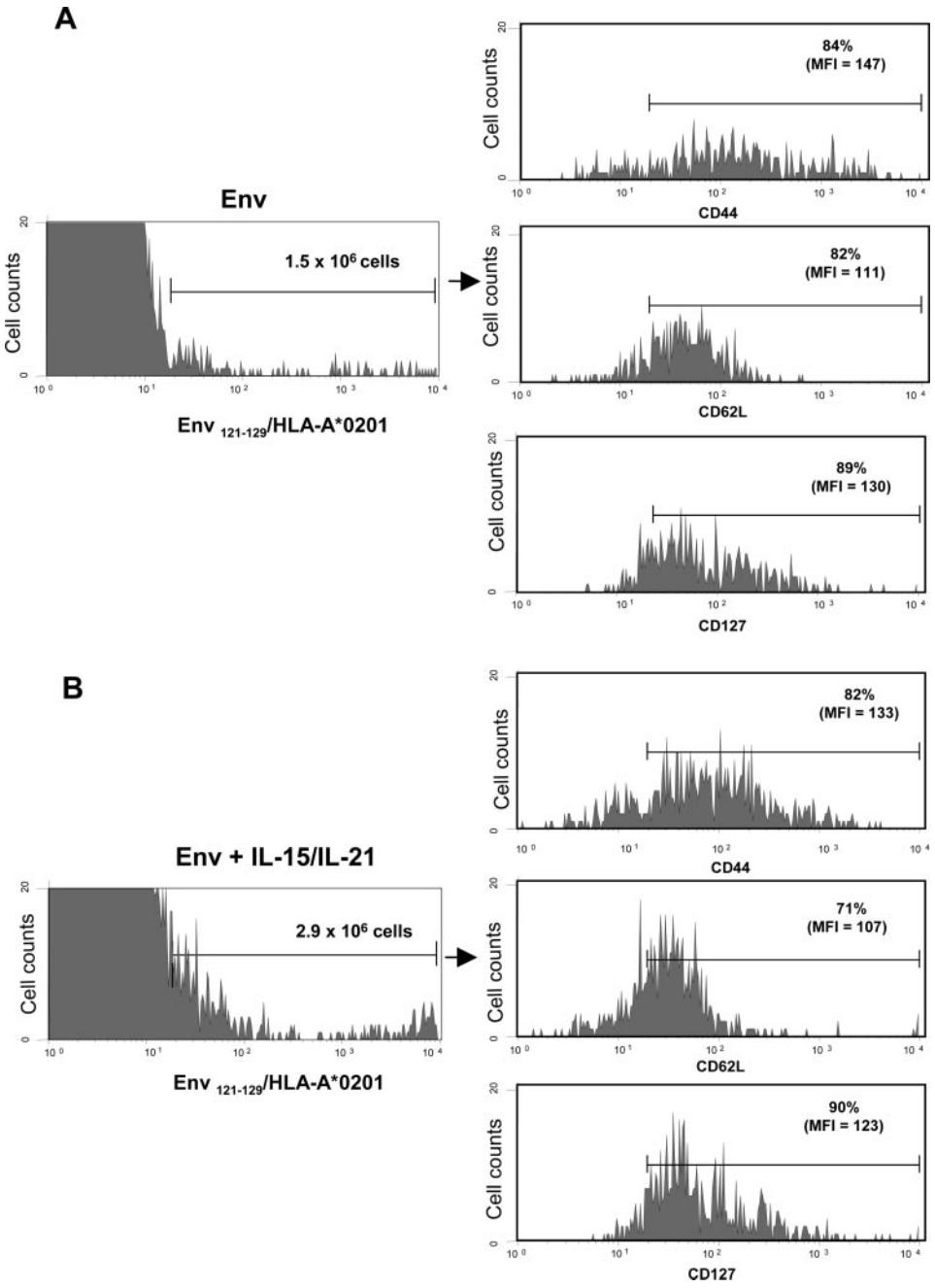

FIGURE 5. Expression of CD44, CD62L, and CD127 Ags on the cell surface of $Env_{121–129}$/HLA-A*0201 tetramer$^+$CD8$^+$ splenocytes in the HLA-A2/K^b transgenic mice immunized with the gp140ΔCFI$_{HxB2/89.6}$ DNA vaccine alone or in combination with *IL-15* and *IL-21* genes. *A*, The HLA-A2/K^b-transgenic mice were immunized three times with the gp140ΔCFI$_{HxB2/89.6}$ DNA vaccine. The phenotypic analysis of splenocytes was performed on 5 wk after the last booster immunization. *B*, The gp140ΔCFI$_{HxB2/89.6}$-immunized HLA-A2/K^b-transgenic mice were injected with *IL-15* and *IL-21* genes as described in *Materials and Methods*. The phenotypic analysis of splenocytes was performed on 5 wk after the last booster immunization. Histograms depict vaccine-induced CD8$^+$ T cells gated first for lymphocytes by forward scatter (FSC) and side scatter (SSC) and then for $Env_{121–129}$ peptide-specific CD8$^+$ T cells by tetramer and anti-CD8 mAb staining. Background staining was assessed using an isotype control Ab. The percent and mean fluorescent intensity (MFI) of CD44, CD62L, and CD127 molecules on $Env_{121–129}$ peptide-specific CD8$^+$ T cells are given. Arrows indicate the source of cells in the histograms. Data are from one representative experiment of three performed.

We next evaluated the effect of IL-15 and IL-21 expression vectors on the gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced effector function of $Env_{121–129}$/HLA-A*0201 tetramer$^+$ CD8$^+$ T cell responses during challenge with vBD3. The experiments were conducted in the HLA-A2/K^b mice 5 wk after the last immunization with the gp140ΔCFI$_{HxB2/89.6}$ construct. The main purpose of this study was to determine whether the vaccinated and cytokine-treated mice harbor increased numbers of $Env_{121–129}$ epitope-specific CD8$^+$ T cells that are capable of responding to the vBD3 virus. The frequency of $Env_{121–129}$ peptide-specific CD8$^+$ T cells in the spleen was determined 5 days after the challenge with vBD3 by staining with $Env_{121–129}$/HLA-A*0201 tetramer. Fig. 6*A* shows that at the peak of the viral infection, $Env_{121–129}$/HLA-A*0201 tetramer-binding CD8$^+$ T cells were readily detectable in gp140ΔCFI$_{HxB2/89.6}$-immunized mice with the numbers ranging from 1.7×10^6 to 2.2×10^6 cells. Consistent with the vaccine-induced Env-specific CTL responses, the numbers of $Env_{121–129}$ peptide-specific cells were augmented over 2-fold in the immunized mice that were treated with the plasmid-encoded IL-15 and IL-21 compared with the untreated counterparts ($4.9 \pm 0.17 \times 10^6$ cells vs $1.9 \pm 0.24 \times 10^6$ cells, $p = 0.02$; Fig. 6*B*). Additional analyses revealed that at the peak of the response to vBD3 challenge, the majority of tetramer-specific cells in both groups of mice had reduced expression of CD127 Ag (Fig. 6, *A* and *B*, *right panel*). In contrast, CD44 was highly expressed on all $Env_{121–129}$/HLA-A*0201 tetramer-binding CD8$^+$ T cells, whereas CD62L expression was present on ~30% of these cells. Consistent with the effector phenotype of the vaccine-induced CD8$^+$ T cells, over 85% of tetramer-positive cells expressed intracellular IFN-γ (Fig. 6, *A* and *B*, *right panel*). The numbers of IFN-γ-producing $Env_{121–129}$/HLA-A*0201-positive cells increased after treatment with *IL-15* and *IL-21* genes compared with those measured in mice that were vaccinated with the gp140ΔCFI$_{HxB2/89.6}$ vector only ($3.8 \pm 0.4 \times 10^6$ cells vs $1.3 \pm 0.2 \times 10^6$ cells, $p = 0.03$).

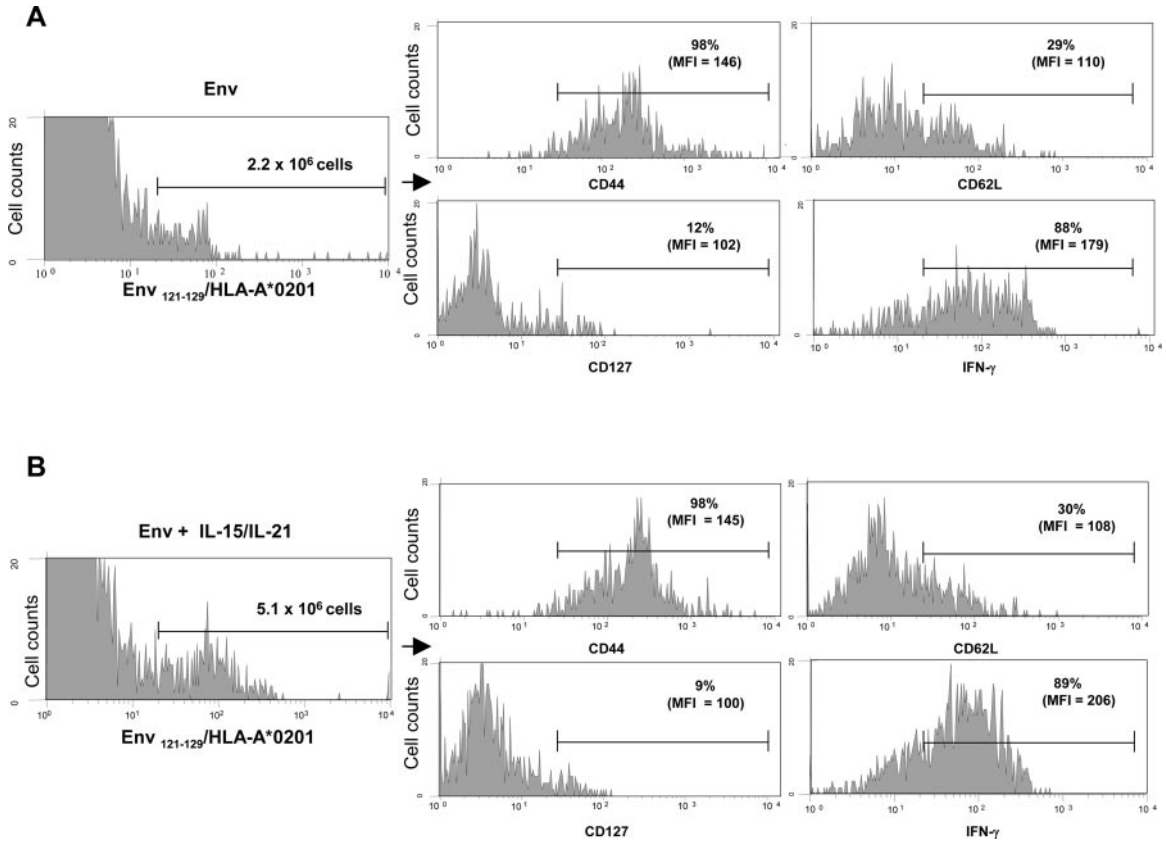

FIGURE 6. IL-21 acts synergistically with IL-51 in expanding the vaccine-induced $CD8^+$ T cell responses to the subdominant $Env_{121–129}$ epitope during vBD3 challenge. *A*, The phenotypic analysis of $Env_{121–129}$ peptide-specific $CD8^+$ T cells in splenocytes of the HLA-A2/K^b transgenic mice immunized with $gp140\Delta CFI_{HxB2/89.6}$ DNA vaccine alone was performed on day 5 of vBD3 challenge. *B*, The effect of coimmunization with *IL-15* and *IL-21* genes on the expansion of the vaccine-induced $CD8^+$ T cell responses to the subdominant $Env_{121–129}$ epitope was analyzed 5 days after vBD3 challenge. Histograms depict vaccine-induced $CD8^+$ T cells gated first for lymphocytes by forward scatter (FSC) and side scatter (SSC) and then for $Env_{121–129}$ peptide-specific $CD8^+$ T cells by tetramer and anti-CD8 mAb staining. Background staining was assessed using an isotype control Ab. The percent and MFI of CD44, CD62L, and CD127 on $Env_{121–129}$ peptide-specific $CD8^+$ T cells are given. Arrows indicate the source of cells in the histograms. The results are representative of four independent experiments.

Protection against challenge with vBD3 vaccinia virus depends on the existence of $CD8^+$ T cells

To examine whether *IL-15* and *IL-21* gene delivery exerts a regulatory effect on the $gp140\Delta CFI_{HxB2/89.6}$ vaccine-induced T cell responses in vivo, we performed depletion experiments of the vaccine-induced $CD8^+$ and $CD4^+$ T cells during the vBD3 challenge study 5 wk after the third immunization. BALB/c mice that were immunized with the $gp140\Delta CFI_{HxB2/89.6}$ vaccine alone or in the presence of IL-15 and IL-21 plasmids were treated with anti-CD4 (clone GK 1.5) or anti-CD8 (clone 53–6.72) mAb. An additional group of the immunized mice was depleted of NK cells, and a control group was treated with rat IgG Ab. As shown in Table I, depletion of either $CD4^+$ T cells or NK cells had no substantial effect on the vBD3 replication in ovaries of $gp140\Delta CFI_{HxB2/89.6}$-immunized mice as the difference in viral titers did not exceed 10% of that measured in animals treated with rat IgG Ab. In contrast, depletion of $CD8^+$ T cells almost completely eliminated the vaccine-induced protection as the vBD3 titer in these mice was similar to that measured in animals treated with rat IgG or challenged with vSC8 virus. Similarly, depletion of $CD8^+$ T cells in mice immunized with the $gp140\Delta CFI_{HxB2/89.6}$ construct in the presence of IL-15 and IL-21 plasmids almost completely eradicated Env-specific protective responses against vBD3. Table I shows that the vBD3 titer increased from 0.3 ± 0.2 $\log_{10}$ PFU in the immunized mice treated with rat IgG to 7.3 ± 0.6 $\log_{10}$ PFU in animals receiving CD8-specific mAb at the time of the challenge ($p < 0.0001$). Conversely, depletion of $CD4^+$ T cells and NK cells had <25% effect on viral replication (Table I), indicating that the synergistic benefit provided by IL-15 and IL-21 treatment was largely dependent on the presence of Env-specific $CD8^+$ T cells.

IL-15 and IL-21 partially replace $CD4^+$ T cell help in the generation of Env-specific $CD8^+$ T cells

Based on the previous observation that coimmunization with a plasmid-delivered IL-15 results in enhanced function and longevity of $CD8^+$ T cells that are partially independent of $CD4^+$ T cell help (72), we tested whether *IL-15* and *IL-21* genes would enhance the vaccine-induced protection against vBD3 vaccinia virus challenge in mice that were partially depleted of $CD4^+$ T cells. In these studies, BALB/c mice were injected i.p. with anti-CD4 mAb to deplete $CD4^+$ T cells before vaccination with the $gp140\Delta CFI_{HxB2/89.6}$ plasmid. This treatment continued every 5 days during the entire period of three immunizations and until the challenge with vBD3 or vSC8 vaccinia virus performed 1 mo later. Additional groups of mice were depleted of $CD8^+$ T cells by a similar treatment with anti-CD8 mAb. Analyses of the numbers of $CD4^+$ and $CD8^+$ T cells in the periphery by flow cytometry revealed that they were kept below 8% for the duration of the study. In BALB/c mice that maintained both $CD4^+$ and $CD8^+$ T cells during immunization with the $gp140\Delta CFI_{HxB2/89.6}$ construct only,

Table I. *Effect of Ab treatment*[a]

Env Vaccine	Adjuvants	Challenge[b]	Treatment[c]	Viral Titer[d] (log_{10}/ovaries)	Protection[e] (Δlog_{10})	Protection[e] (%)
gp140ΔCFI$_{HxB2/89.6}$		vSC8	None	7.8 ± 0.6		
		vBD3	IgG	6.0 ± 0.2	1.8	23
		vBD3	Anti-CD4	6.3 ± 0.4	1.5	19
		vBD3	Anti-CD8	7.5 ± 0.5	0.3	4
		vBD3	Anti-asialo GM1	6.6 ± 0.7	1.2	15
gp140ΔCFI$_{HxB2/89.6}$	IL-15/IL-21	vSC8	None	8.2 ± 0.7		
	IL-15/IL-21	vBD3	IgG	0.3 ± 0.2	7.9	96
	IL-15/IL-21	vBD3	Anti-CD4	1.5 ± 1.0	6.7	82
	IL-15/IL-21	vBD3	Anti-CD8	7.3 ± 0.6	0.9	11
	IL-15/IL-21	vBD3	Anti-asialo GM1	2.4 ± 1.2	5.8	71

[a] Effect of Ab treatment on replication of vBD3 vaccinia virus in ovaries of mice immunized with the gp140ΔCFI$_{HxB2/89.6}$vaccine in the presence, absence, or combination of *IL-15* and *IL-21* genes.

[b] BALB/c mice were immunized with the gp140ΔCFI$_{HxB2/89.6}$ construct in the presence, absence, or combination of IL-15 and IL-21 plasmids. Five weeks after the third immunization, mice were challenge i.p. with 2×10^7 PFU of vBD3 or vSC8 virus. The viral titer in ovaries was analyzed 5 days after the challenge.

[c] To evaluate the contribution of CD8 and CD4 T cells to protection against vBD3 challenge, the immunized mice were treated i.p. with 100 μg of anti-CD4 mAb (clone GK1.5), anti-CD8 mAb (clone 53-6.72), rat IgG, or 25 μl of anti-asialo GM1 (GA1) rabbit serum on days 3 and 1 before and day 1 after the challenge with vBD3.

[d] The rVV titers were determined in ovaries of three mice per group (mean log_{10} ± SD).

[e] Protection is reduction of vBD3 titers in ovaries (Δlog_{10} PFU or %) compared with the value for the control group challenged with vSC8.

the vBD3 titer in ovaries was ~100-fold lower than that in vSC8-challenged animals (Fig. 7*A*; $p < 0.05$). In contrast, CD4 T cell-depleted mice that were immunized with the gp140ΔCFI$_{HxB2/89.6}$ vaccine resulted in lack of protective immunity against vBD3 challenge, supporting the need for $CD4^+$ Th cells during priming in the generation of functional $CD8^+$ effector T cells. Also, the vaccine-primed and $CD8^+$ T cell subset-depleted mice were unable to inhibit replication of the vBD3 vaccinia virus (Fig. 7*A*).

The same experiments repeated in mice that were immunized with the combined regimen of gp140ΔCFI$_{HxB2/89.6}$ vaccine together with *IL-15* and *IL-21* genes consistently resulted in a lower titer of vBD3 compared with vSC8-challenged animals ($p < 0.0001$, Fig. 7*B*). In the presence of IL-15 and IL-21 treatment, we observed modest levels of protection after coimmunization of CD4 T cell-depleted mice with gp140ΔCFI$_{HxB2/89.6}$ vaccine (Fig. 7*B*). Interestingly, the viral titers of vBD3 in ovaries of these mice were similar to those observed in gp140ΔCFI$_{HxB2/89.6}$-immunized parental group of mice (Fig. 7*A*). Depletion of $CD8^+$ T cells almost completely eliminated the vaccine-induced protection as the vBD3 titer in these mice was similar to that measured in vSC8-challenged animals. Taken together, these data indicate that in the partial presence of $CD4^+$ T cells, the plasmid-delivered IL-15 and IL-21 can augment protective immunity almost to a normal level.

IL-15 and IL-21 enhance humoral responses and the effector function of Env-specific Abs in gp140ΔCFI$_{HxB2/89.6}$-immunized mice

Based on the previous studies which demonstrated that IL-21 regulates humoral immunity by promoting B cell maturation during a productive T lymphocyte-dependent B cell response while favoring growth arrest and apoptosis for nonspecifically or inappropriately activated B cells (73, 74), we sought to examine the effect of IL-21 expression vector delivered alone or in combination with *IL-15* gene on the gp140ΔCFI$_{HxB2/89.6}$ vaccine-induced Ab responses. BALB/c mice were immunized three times with the gp140ΔCFI$_{HxB2/89.6}$ construct in the presence, absence or combination of *IL-15* and *IL-21* genes. In all experiments, IL-15 was delivered together with the vaccine, whereas IL-21 expression vector was injected 5 days later. The titers of Env-specific IgM Abs in sera of gp140ΔCFI$_{89.6}$-immunized mice were analyzed after each immunization. The ELISA with plates coated with the Env glycoprotein was used to measure the end-point titers and isotypes of the vaccine-induced serum Abs collected 3 wk after each booster immunization. As shown in Fig. 8*A*, detectable levels of Env-specific IgM Ab responses were observed already after the first immunization with the gp140ΔCFI$_{HxB2/89.6}$ vector with only small differences among mice vaccinated in the presence or absence of IL-15 and IL-21 plasmids. The titers of IgM Abs in gp140ΔCFI$_{HxB2/89.6}$-immunized mice increased after the second immunization in all groups of the immunized mice ($p < 0.05$), but showed only moderate enhancement after the third immunization. Moreover, similar anti-Env IgM Ab responses were observed in all immunization groups after each booster vaccination, suggesting that IL-15 and IL-21 administered either alone or in combination had no substantial effect on the gp140ΔCFI$_{HxB2/89.6}$-induced IgM Ab level. In contrast, coimmunization with IL-15 and IL-21 expression vectors augmented Env-specific IgG Ab responses, which were most prominent after the second and third booster immunization with the gp140ΔCFI$_{HxB2/89.6}$ vaccine (Fig. 8*B*). We also observed that IL-21 preferentially augmented Env-specific IgG1 Abs and had a modest effect on gp140ΔCFI$_{HxB2/89.6}$-induced IgG2, whereas IL-15 plasmid increased Env-specific IgG2a as well as IgG2b Ab production (Fig. 8*C*). None of the cytokines affected production of Env-specific IgG3 Ab responses, which remained at a low level in all groups of the immunized mice.

The functional significance of IL-21- and IL-15-mediated increases in Env-specific IgG Ab responses was further investigated by analyzing the ability of sera derived from mice immunized three times with the gp140ΔCFI$_{HxB2/89.6}$ construct in the presence, absence or combination of IL-15 and IL-21 plasmids to mediate ADCC and CDC of Env-expressing target cells. In the ADCC assay, ^{51}Cr-labeled target cells infected with vBD3 virus were incubated with a serial dilution of sera collected 3 wk after the last immunization in the presence of NK-cell-enriched splenocytes from naive BALB/c mice. Fig. 9*A* shows that the highest level of lysis was obtained with sera from gp140ΔCFI$_{HxB2/89.6}$-immunized mice treated with both IL-15 and IL-21 plasmids. In these mice, the cytolytic responses against Env-expressing cells were still detectable at the serum dilution of 1/270, whereas they were at a

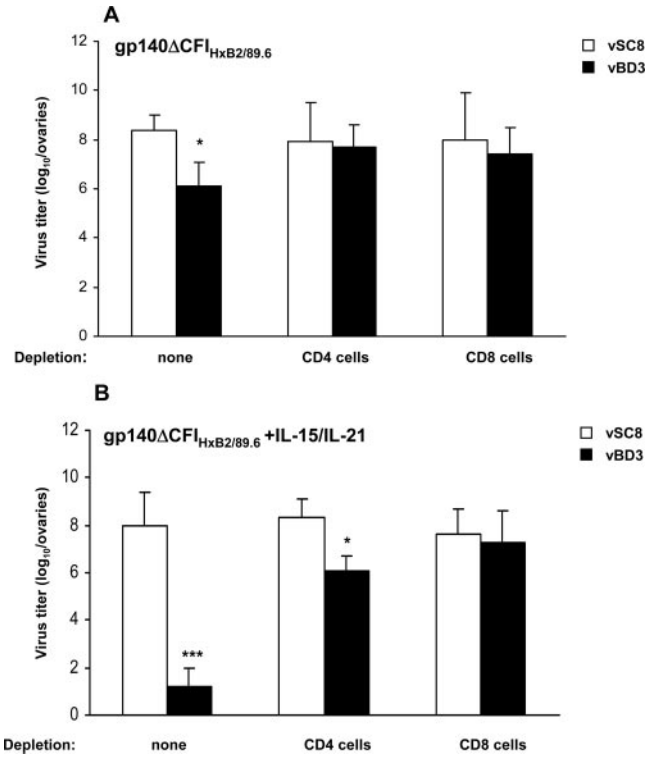

FIGURE 7. The generation of functional $CD8^+$ T cells in $gp140\Delta CFI_{HxB2/89.6}$-immunized mice requires $CD4^+$ T cells during priming and is partially restored by coimmunization with *IL-15* and *IL-21* genes in a model of $CD4^+$ Th cell depletion. *A*, BALB/c mice were depleted of $CD4^+$ T cells before vaccination with the $gp140\Delta CFI_{HxB2/89.6}$ construct by i.p. injection of anti-CD4 mAb. Additional groups of mice were depleted of $CD8^+$ T cells by treatment with anti-CD8 mAb. The Ab treatment continued every 5 days during the entire period of three immunizations and until the challenge studies with vBD3 (■) and vSC8 (□) vaccinia viruses performed 1 mo later. *B*, BALB/c mice were depleted of $CD4^+$ T cells before vaccination with the $gp140\Delta CFI_{HxB2/89.6}$ construct and treatment with the combination of *IL-15* and *IL-21*genes by i.p. injection of anti-CD4 mAb. In all experiments, IL-15 was delivered together with the vaccine, whereas IL-21 expression vector was injected 5 days later. Additional groups of mice were depleted of $CD8^+$ T cells by treatment with anti-CD8 mAb. The vaccinia titers are presented as the mean $\log_{10} \pm$ SD (error bars) of PFU per ovaries of four mice per group. *, $p < 0.05$; ***, $p < 0.0001$.

background level with vSC8-infected targets. The CDC assay revealed comparable levels of lysis with sera from mice coimmunized with IL-15 plasmid and those treated with both cytokines (Fig. 9*B*), possibly due to similar Env-specific IgG2 Ab responses.

Discussion

The generation of immunological memory against clinically relevant HIV-1 isolates is the end result of a productive immune response and is the ultimate goal for an effective AIDS vaccine. As the size of naive and memory T cell pools is tightly regulated, at least in part, by growth and survival signals conferred by γ_c-dependent cytokines (75, 76), it should therefore be possible to use these cytokines in vivo to increase the immunogenicity of HIV-1 vaccines. In these studies, we presented data on a synergistic effect of two γ_c cytokines such as IL-21 and IL-15 in enhancing the level and longevity of protective immune responses induced by the $gp140\Delta CFI_{HxB2/89.6}$ DNA vaccine. Our results are in agreement with the previous studies which showed that IL-15 and IL-21 are capable of promoting potent augmentation of $CD8^+$ T cell activation and survival (40, 77, 78). Importantly, IL-15- and IL-21-mediated enhancement of T cell effector differentiation in response to stimulation with a subdominant Ag would indicate that these cytokines could be clinically useful for enhancing the breadth of immune responses to T cell-based vaccines. In support of this hypothesis, our results in $gp140\Delta CFI_{HxB2/89.6}$-immunized HLA-$A2/K^b$ transgenic mice demonstrate that IL-21 and IL-15 expression constructs synergistically promoted expansion of memory $CD8^+$ T cells to the subdominant $Env_{121-129}$ epitope.

The current paradigms hold that memory $CD8^+$ T cells represent highly fit cells derived from the effector T cell population (79–81), thus leading to the prediction that therapies which augment T cell effector pools may also augment T cell memory pools (82, 83). Our results are consistent with this hypothesis as well as the most recent studies which show that rIL-21 cooperates with IL-15 in achieving the expansion of tumor-specific $CD8^+$ T cells associated with regression of B16 melanoma (41). The present study further extends these observations and demonstrates for the first time a synergistic action of *IL-15* and *IL-21* genes in augmenting the efficacy of HIV-1 Env-specific DNA vaccine through induction of durable Env-specific immune responses which were protective against vBD3 vaccinia virus challenge.

Similar to the results obtained by Zeng et al. (41), we found that the effect of *IL-21* gene on the vaccine-induced immune responses is dose dependent and optimal at 20 μg of plasmid DNA/mouse. Interestingly, injection of the IL-21-encoded plasmid 5 days after specific vaccination induced the highest and long-lasting protection, whereas delivery of the plasmid simultaneous with specific vaccination produced suboptimal benefit for the enhancing of specific immunity. These findings are consistent with previous reports in which IL-21 addition led to an optimal augmentation of the specific T cell responses when provided after Ag priming (40, 84). They are also in an agreement with the recent data indicating that IL-21 expression during challenge with HSV-2 or lymphocytic choriomeningitis virus is elevated on days 5 and 7 and coincide with the onset of the adaptive immune response (85). Altogether, these studies suggest that IL-21 promotes adaptive immunity by regulating the expansion and/or restoration of vaccine-induced T cell responses, and only marginally affects Ag priming of naive cells. This possibility is also supported by another study which demonstrated that IL-21 protein used at concentrations of 20 and 100 ng/ml blocked the LPS-induced maturation of dendritic cells in vitro (86). Similarly, we found that administration of recombinant mouse IL-21 protein (>10 μg per injection) at the time of the DNA vaccination and for 7 consecutive days reduced the level of Env-specific T cell responses and protection (data not shown), suggesting that when delivered at suboptimal concentrations before vaccines, IL-21 may diminish Ag presentation. These results together with those of previous studies which showed that IL-15 regulates naive T cell viability and promotes the maintenance of memory T cells (18), suggest that a dual approach of expanding the pool of activated $CD8^+$ T cells with IL-15 together with promoting their differentiation with IL-21 may augment the vaccine-specific immune responses.

Another major impediment to the development of successful vaccines against HIV-1, which requires broadly reactive cellular immune responses for overcoming mutations to target Ags as well as anergy and/or suppression due to chronic Ag overload, is the limited number of vaccine modalities and adjuvants capable of eliciting $CD4^+$ and $CD8^+$ T cell responses. It is believed that $CD8^+$ T cell responses are important for controlling HIV-1 infection and slowing disease progression. Although the exact function of HIV-1-specific $CD8^+$ T cells in this process has not been fully elucidated, a correlation has been established between long-term

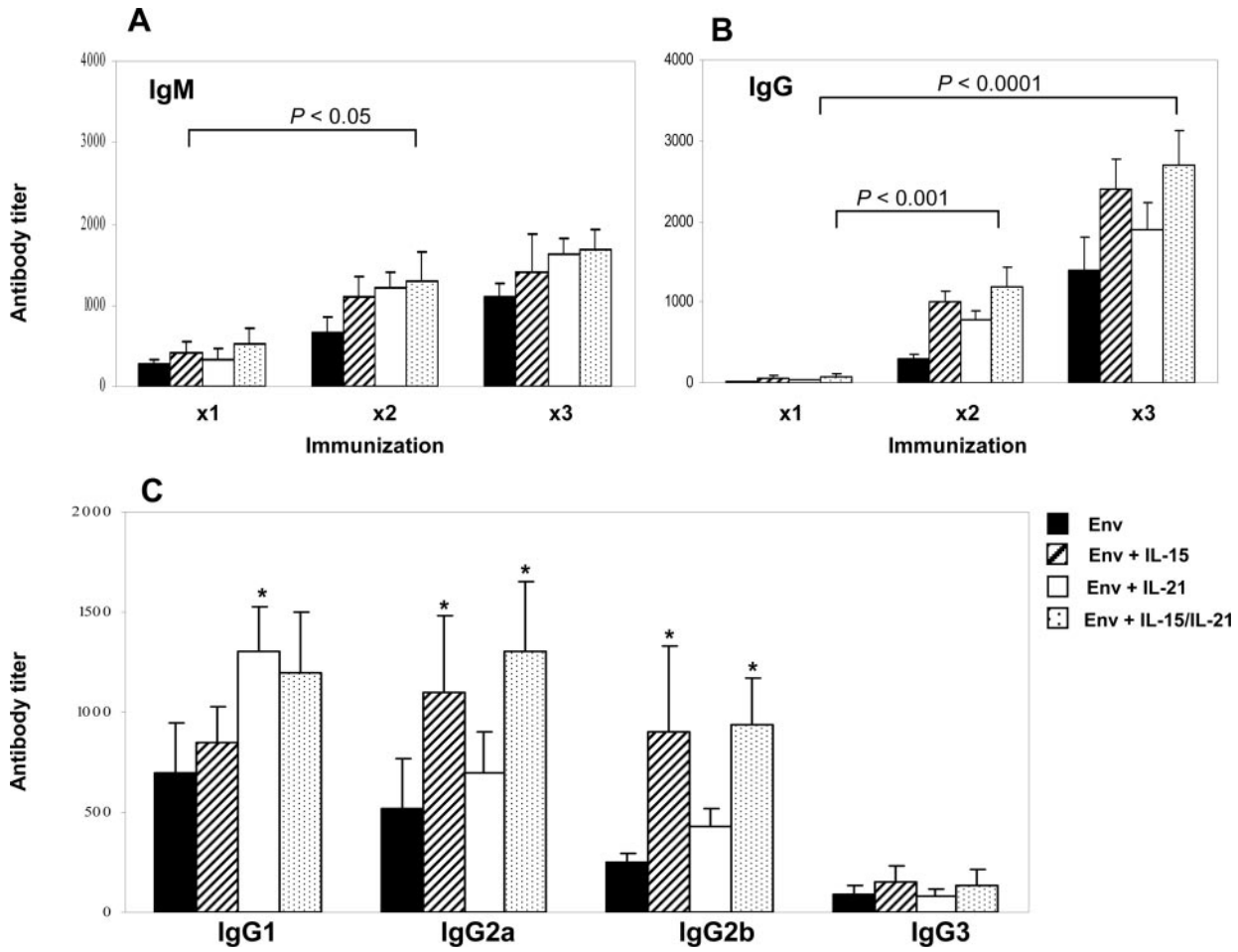

FIGURE 8. Env-specific Ab responses induced by vaccination of BALB/c mice with the gp140ΔCFI$_{89.6}$ construct in the presence, absence, or combination of *IL-15* and *IL-21* genes. *A*, BALB/c mice were immunized three times with the gp140ΔCFI$_{HxB2/89.6}$ construct in the presence, absence, or combination of IL-15 and IL-21 genes. In all experiments, IL-15 was delivered together with the vaccine, whereas IL-21 expression vector was injected 5 days later. The titers of Env-specific IgM Abs in sera of gp140ΔCFI$_{89.6}$-immunized mice were determined after each immunization by ELISA using alkaline phosphatase-conjugated secondary Abs. *B*, The titers of Env-specific IgG Abs were analyzed in the same sera of the gp140ΔCFI$_{89.6}$ construct-immunized mice. *C*, The titers of Env-specific IgG subclasses were determined in sera after the third immunization with the gp140ΔCFI$_{89.6}$ construct in the presence, absence, or combination of *IL-15* and *IL-21* genes. Results are presented as the means ± SD (error bars) of three independent experiments. *, $p < 0.05$.

nonprogression of HIV-1 in seropositive individuals and HIV-1-specific CD8$^+$ T cells to maintain effector T cell activity and control virus. This suggests that understanding the factors that supplement CD4$^+$ T cell function and support CD8$^+$ T cell activity could be important for HIV-1 therapy strategy where CD4 help is compromised. Therefore, the reported ability of IL-15 plasmid to

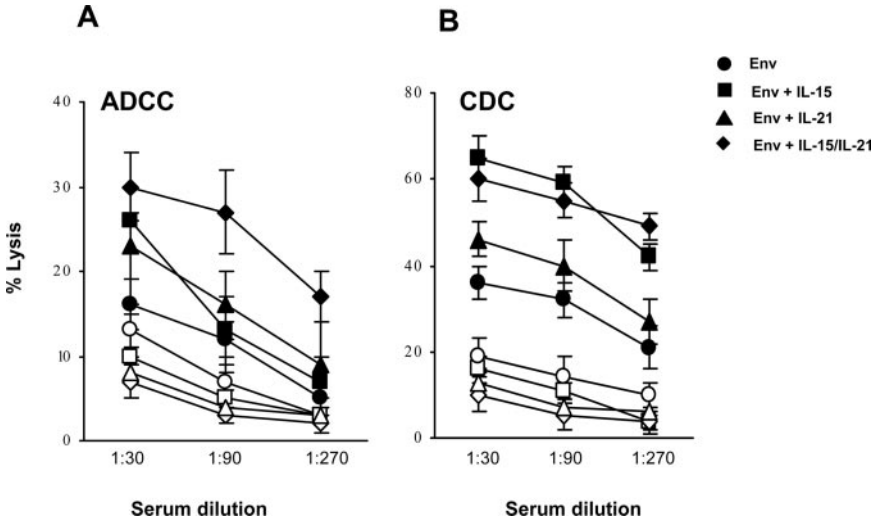

FIGURE 9. ADCC and CDC against Env-expressing cells mediated by sera of mice immunized with the gp140ΔCFI$_{89.6}$ construct in the presence, absence or combination of *IL-15* and *IL-21* genes. *A*, ADCC was measured using vBD3-infected (solid symbols) and vSC8-infected (open symbols) target cells and serial dilutions of sera collected 3 wk after the third immunization with the Env vaccine in the presence, absence, or combination of *IL-15* and *IL-21* genes. *B*, The complement-mediated lysis of vBD3-infected (black symbols) and vSC8-infected (white symbols) target cells was determined using the same sera as for the ADCC assay. All determinations were made in triplicate samples, and the SD was <10%. Results are presented as the means ± SD (error bars) of three independent experiments.

restore $CD8^+$ T cell secondary immune responses to an antigenic DNA plasmid in the partial absence of $CD4^+$ T cells may be useful in contributing to vaccine control of HIV-1 infection (72). Our results that the combination of IL-15 and IL-21 enhanced the vaccine-induced protection against vBD3 vaccinia virus challenge in mice that were partially depleted of $CD4^+$ T cells are supportive of the previous findings. It has been suggested that IL-15 directly activates $CD8^+$ T cells during the priming stage, leading to increased formation of memory cells (72). Alternatively, IL-15 may directly activate APCs, leading to the secretion of IL-12, which subsequently induces IFN-γ to further activate dendritic cells and macrophages and provide costimulation of $CD8^+$ T cells (72). Although these possibilities remain to be examined, we hypothesize that IL-21 may promote differentiation of IL-15-activated $CD8^+$ T cells. In support of this possibility are our preliminary findings in the CD4 knockout mice which showed that coimmunization with *IL-15* and *IL-21* genes provides higher protection than IL-15 alone, whereas IL-21 vector had no substantial effect on the responses. Additional experiments are required to determine the mechanism by which the interaction between IL-15 and IL-21 cytokines would promote beneficial immune responses, especially in a model of CD4 depletion.

To design an effective vaccine, it is important to understand the types of immune responses required to prevent infection or disease progression. Many currently licensed vaccines mediate protection primarily by humoral immune responses, in which Abs bind and neutralize the pathogen. However, for many diseases including AIDS, it can be envisioned to be difficult for vaccines to elicit broadly neutralizing Abs. In such cases, adaptive cellular immune responses together with innate immunity are critical for control of the pathogen and protection against disease progression, although both humoral and cellular immune responses will likely be required for optimal and sustained protection. Consistent with the reported results in a B16 melanoma model (41), our data demonstrate that both IL-15 and IL-21 synergize in promoting the generation and survival of $gp140\Delta CFI_{HxB2/89.6}$ vaccine-induced durable cellular responses capable of eradicating vBD3 infection in association with the expansion of Env-specific $CD8^+$ T cells. Moreover, we also found that combination of IL-15 and IL-21 plasmids increases the cytolytic effector function of humoral responses including ADCC and CDC through augmentation of Env-specific IgG Ab level. This, together with the highlighted importance of IL-21 in inducing long-lasting and effective $CD8^+$ T responses (48, 87–89), suggests that IL-21-mediated immunomodulation warrants further evaluation for HIV-1 vaccine.

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Disclosures

The authors have no financial conflict of interest.

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